

**APPLICATION
FOR
UNITED STATES LETTERS PATENT**

To whom it may concern:

Be it known that

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have invented certain new and useful improvements in

**METHODS FOR REGULATING A LYMPHOCYTE-MEDIATED IMMUNE
RESPONSE BY BLOCKING COSTIMULATORY SIGNALS AND BLOCKING
LFA-1 MEDIATED ADHESION IN LYMPHOCYTES**

of which the following is a full, clear and exact description.

METHODS FOR REGULATING A CELL-MEDIATED IMMUNE RESPONSE BY BLOCKING LYMPHOCYTIC SIGNALS AND BY BLOCKING LFA-1-MEDIATED ADHESION

- 5 This application claims the benefit of the filing date of U.S. Serial Nos. 60/210,671, filed June 9, 2000. The contents of the foregoing application are incorporated by reference in their entirety, into the present application.

Throughout this application various publications are referenced. The disclosures of these
10 publications, in their entireties, are hereby incorporated by reference into this application, in order to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

- 15 The present invention relates to improved methods for regulating cell-mediated immune responses by disrupting at least three interactions between cell surface molecules and their natural ligands.

BACKGROUND OF THE INVENTION

- 20 Acquired (specific) immunity is a stratagem used by a body to expand the repertoire of options available to combat antigenic challenge. Acquired immunity is mediated by lymphocytes, which are produced in the bone marrow by hematopoiesis. Activation of lymphocyte-mediated immunity in response to antigen recognition and binding result in
25 activation of the two major subpopulations of lymphocytes: B lymphocytes (B-cells) and T lymphocytes (T-cells).

- T- and B-cells are activated in an interdependent fashion. After antigenic challenge to a host, some host cells such as B-cells, macrophages and dendritic cells capture,
30 internalize, and process the antigen for presentation on the cell surface. Then, after recognition and binding of the presented antigen by T-cells (specifically a subset of T-cells known as T-helper cells), the T-cells activate other T-cells as well as B-cells. In

turn, activated B-cells stimulate resting T-cells. The complicated interactions between B- and T-cells that regulate their activities are known to be mediated by several cell surface molecules.

- 5 Regulation of T-cell activation following recognition and binding of allo-antigen has been shown to require two distinct molecular signals mediated by cell surface molecules. The first signal is provided to the T-cell via antigen recognition and binding of the T-cell receptor, while the second signal is thought to be provided by recognition and binding of one or more of several putative receptor molecules on the surface of the T-cell by their
- 10 ligands (1). To date, the most likely of these cell surface receptor molecules have been CD28 (2-5), CTLA4 (60-61) and CD154 (also known as CD40L or gp39) (6-9). The CD28 molecule is expressed on nearly all CD4+ T cells and approximately 50% of CD8+ T cells (4). The ligands for CD28 have been shown to be the B7-1 and B7-2 (also known as CD80 and CD86, respectively) molecules expressed on the surface of antigen
- 15 presenting cells such as B cells (4; 5; 10). Interaction between CD28 and the B7 molecules stimulates the activation of T-cells.

The B7 molecules are also ligands for the cell surface receptors, CTLA4, which are present on activated T-cells. Interaction between CTLA4 and B7 induces a state of

20 anergy in T-cells, counteracting CD28/B7 induced activation of T-cells.

The interaction of CD28 and/or CTLA4 with B7 ligands can be efficiently blocked by soluble CTLA4Ig (DNA encoding CTLA4Ig was deposited on May 31, 1991 with American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA

25 20110-2209, with ATCC identification number 68629; and CTLA4Ig-24, a Chinese Hamster Ovary (CHO) cell line expressing CTLA4Ig was deposited on May 31, 1991 with ATCC identification number CRL-10762), a fusion protein with a higher avidity for B7-1 and B7-2 than CD28 (11-13). Numerous studies have demonstrated that blockade of this receptor pathway leads to inhibition of antigen activation of T-cells both *in vitro*

30 (13; 14) and *in vivo* (12; 15; 16).

The CD154 molecule is predominantly expressed on activated T-cells while CD40 is expressed on antigen presenting cells such as B cells. The interaction between CD154 and CD40 activates B-cells and more T-cells. Blockade of the CD40/CD154 pathway by monoclonal antibodies has also proven to be an effective method of inhibiting lymphocyte-mediated immune responses *in vitro* (17; 18) and *in vivo* (19-21).

Although CD28, CTLA4 and CD154 have been the most studied lymphocyte receptors, other cell surface molecules have been implicated in the cell regulation process. Some of these molecules include 4-1BB, ICOS, CD99 and several adhesion molecules from the integrin family, such as Lymphocyte function associated antigen-1 (LFA-1) (22-33).

The LFA-1 molecule is formed by the combination of the integrin α L subunit (CD11a) with the integrin β 2 subunit (CD18). LFA-1 is expressed on numerous leukocyte cell types including lymphocytes (e.g. T-cells), granulocytes, monocytes, macrophages, etc., and has been described in detail as mediating cell-cell and cell-matrix interactions via its interaction with various ligands, including molecules from the ICAM family (e.g. ICAM-1, ICAM-2 and ICAM-3). Other ligands of the LFA-1 β 2 subunit (CD18) include α -actinin, filamin, and cytohesin-1. The function of LFA-1 as an adhesion molecule, augmenting the interaction between LFA-1-positive cells and LFA-1 binding ligands such as ICAM-1 on leukocytes, epithelial cells and endothelium, has been long recognized; however, more recently, evidence has emerged implicating LFA-1 in the signaling process of T cells following antigen recognition (26; 27; 34-38).

Following allogeneic organ or tissue transplantation, cell-mediated graft rejection is still a major obstacle to successful long-term graft survival. The use of monoclonal antibodies that block the LFA-1/ICAM-1 interaction has been shown to prolong graft survival (39-44). In addition, agents that block lymphocytic interactions such as CTLA4Ig and anti-CD40/CD154 monoclonal antibodies, have recently been demonstrated to be efficacious against the graft rejection process in multiple animal models including non-human primates (14; 45-51). Furthermore, combining both CD28 and CD40/CD154 blockade dramatically improved graft survival leading to long-term graft acceptance and allogeneic

hypo-responsiveness in some animal models of transplantation (52-54). Yet, other models of allogeneic transplantation, such as BALB/c->C57BL/6 murine skin transplants, have been shown to be resistant to CD28+CD154 blockade (55), suggesting that other signaling pathways may be involved in allograft recognition.

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Presently, there exists a need to provide ways to regulate cell-mediated immune responses after antigen presentation, for example to suppress graft (e.g., allograft) rejection after transplantation of tissues, so as to increase the survival rate of the transplanted tissue.

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The inhibition of immune responses resulting from the blockade of CD28/B7, CTLA4/B7 and/or CD40/CD154 signals is potent, but incomplete in some cases. The results presented herein demonstrate that blockade of these pathways, in addition to blockade of the LFA-1 pathway, unexpectedly enhances graft survival and regulates the cell-mediated immune response to antigen presentation.

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SUMMARY OF THE INVENTION

The invention disclosed herein provides methods for regulating cell-mediated immune responses comprising blocking the interaction of cell surface molecules such as CD28, CTLA4, B7, CD40, CD154 and adhesion molecules such as LFA-1, with their natural ligands. The invention herein involves the discovery that blockade of these molecules provides improved methods to promote long-term survival of transplants. Addition of an agent directed against LFA-1 greatly enhances allogeneic graft survival in mice treated with an agent directed against B7 (e.g. soluble CTLA4 molecules) and an agent against CD154 (e.g. anti-CD154 monoclonal antibody) for both murine skin and cardiac transplants. Use of a combination of three agents, for example, directed against each of CD28/CTLA4/B7, CD40/CD154 and LFA-1/ICAM pathways, respectively, also enhances murine skin graft survival. The present invention also provides methods for regulating immune system diseases such as those associated with allograft transplantation.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph showing the effect of soluble CTLA4-Ig and monoclonal antibodies MR1 and anti-LFA-1 on skin graft rejection rates, as described in Example 1, *infra*.

Figure 2 is a graph showing the effect of soluble CTLA4-Ig and monoclonal antibodies MR1 and anti-LFA-1 on heart graft rejection rates, as described in Example 2, *infra*.

Figure 3 show the percentage of myocardium remaining after transplantation and therapy with soluble CTLA4-Ig and monoclonal antibodies MR1 and anti-LFA-1, in a murine heterotropic heart transplant model, as described in Example 2, *infra*.

Figure 4 shows inflammation severity scores after transplantation and therapy with soluble CTLA4-Ig and monoclonal antibodies MR1 and anti-LFA-1, in a murine heterotropic heart transplant model, as described in Example 2, *infra*.

Figure 5 shows the amino acid and nucleic acid sequence of human CTLA4Ig with a leader sequence attached to the N-terminus of the molecule, as described in Example 3, *infra*.

Figure 6 shows the amino acid and nucleic acid sequence of human L104EA29YIg with a leader sequence attached to the N-terminus of the molecule, as described in Example 3, *infra*.

Figure 7 are an SDS gel (FIG. 7A) for CTLA4Ig (lane 1), L104EIg (lane 2), and L104EA29YIg (lane 3A); and size exclusion chromatographs of CTLA4Ig (FIG. 7B) and L104EA29YIg (FIG. 7C).

Figures 8A and 8B illustrate a ribbon diagram of the CTLA4 extracellular Ig V-like fold generated from the solution structure determined by NMR spectroscopy. FIG. 8B shows

an expanded view of the S25-R33 region and the MYPPPY region indicating the location and side-chain orientation of the avidity enhancing mutations, L104 and A29.

Figures 9A & 9B illustrate data from FACS assays showing binding of L104EA29YIg, L104EIg, and CTLA4Ig to human CD80- or CD86-transfected CHO cells as described in Example 3, *infra*.

Figures 10A & 10B depicts inhibition of proliferation of CD80-positive and CD86-positive CHO cells as described in Example 3, *infra*.

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Figures 11A & 11B shows that L104EA29YIg is more effective than CTLA4Ig at inhibiting proliferation of primary and secondary allostimulated T cells as described in Example 3, *infra*.

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Figures 12A-C illustrate that L104EA29YIg is more effective than CTLA4Ig at inhibiting IL-2 (FIG. 12A), IL-4 (FIG. 12B), and γ -interferon (FIG. 12C) cytokine production of allostimulated human T cells as described in Example 3, *infra*.

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Figure 13 demonstrates that L104EA29YIg is more effective than CTLA4Ig at inhibiting proliferation of phytohemagglutinin- (PHA) stimulated monkey T cells as described in Example 3, *infra*.

DETAILED DESCRIPTION OF THE INVENTION

25 Definitions

As used in this application, the following words or phrases have the meanings specified.

As used herein, "ligand" refers to a molecule that recognizes and binds another molecule.

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As used herein, "regulate" means to inhibit or stimulate a response, for example "regulating a lymphocyte-mediated immune response" means to inhibit or stimulate a lymphocyte associated immune response.

- 5 As used herein, "receptor" means a molecule that, when bound by a ligand, instigates an intracellular pathway cascade leading to an altered cell state. Receptors may be found on several cell types including lymphocytes.

- 10 As used herein, to "block" or "inhibit" a receptor, signal or molecule means to interfere with the activation of the receptor, signal or molecule, as detected by an art-recognized test. For example, blockage of a cell-mediated immune response can be detected by determining enhancement of allogeneic graft survival. Blockage or inhibition may be partial or total.

- 15 As used herein, "inhibit cell-cell" or "cell-matrix adhesion" means to prevent the interaction between a cell surface adhesion molecule and its ligand on another cell or in the extracellular matrix. Examples of adhesion molecules and ligands include, but are not limited to, LFA-1 (also known as CD11a/CD18 or as integrin α L β 2), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18), ICAM-1 (CD54), ICAM-2, ICAM-3, VLA-1, CD44, CD62 (E, L and P), CD106, fibrinogen, α -actinin, filamin and cytohesin-1.
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- As used herein, a "portion" or "fragment" of a molecule means any part of the intact molecule that retains binding activity. For example, a "fragment of CTLA4" or "portion of CTLA4" is the extracellular domain of CTLA4 or segment thereof that recognizes and binds its target, e.g. B7.
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- As used herein a "derivative" is a molecule that shares sequence homology and activity of its parent molecule. For example, a derivative of CTLA4 includes a soluble CTLA4 molecule, or a soluble CTLA4 mutant molecule, having an amino acid sequence at least 70% similar to the extracellular domain of wildtype CTLA4, and which recognizes and binds B7. An example of a soluble CTLA4 molecule is CTLA4Ig (deposited on May 31,
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1991 with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209. ATCC identification number 68629). CTLA4Ig-24, a Chinese Hamster Ovary (CHO) cell line expressing CTLA4Ig was deposited on May 31, 1991 with ATCC identification number CRL-10762. An example of a soluble CTLA4 mutant molecule is L104EA29YIg (deposited on June 19, 2000 with ATCC identification number PTA-2104).

As used herein, "agent" refers to a molecule that is used to regulate a cell-mediated immune response by blocking molecules mediating the immune response.

As used herein, "first agent" is a molecule, or a portion or portions thereof, that recognizes and binds the CD28, CTLA4 or B7 molecules on lymphocytes. For example, "first agent" may be, but is not limited to, soluble CTLA4 molecules (e.g. soluble CTLA4 mutant molecules), soluble CD28 molecules, soluble B7 molecules, anti-CD28 monoclonal antibodies, anti-CTLA4 monoclonal antibodies, or anti-B7 monoclonal antibodies, including fragments or derivatives thereof.

As used herein, "second agent" is a molecule, or a portion or portions thereof, that recognizes and binds CD40 or CD154 (also known as CD40L or as gp39) on lymphocytes. For example, "second agent" may be, but is not limited to, soluble CD40, soluble CD154, anti-CD40 monoclonal antibodies, or anti-CD154 monoclonal antibodies, including fragments or derivatives thereof.

As used herein, "third agent" is a molecule, or a portion or portions thereof, that interferes with LFA-1 adhesion to its ligands such as ICAM-1, ICAM-2, ICAM-3, α -actinin, filamin or cytohesin-1.. For example, "third agent" may be, but is not limited to, an anti-LFA-1 monoclonal antibody, an anti-ICAM-1 monoclonal antibody, an anti-ICAM-2 monoclonal antibody, an anti-ICAM-3 monoclonal antibody, an anti-CD11a monoclonal antibody, an anti-CD18 monoclonal antibody and soluble forms of LFA-1, CD11a, CD18, ICAM-1 (CD54), ICAM-2 or ICAM-3, including fragments or derivatives thereof.

As used herein, "B7" refers to B7 family members including B7-1 (also known as CD80), B7-2 (also known as CD86) and B7-3 molecules that may recognize and bind CD28 and/or CTLA4.

5 As used herein "wild type CTLA4" has the amino acid sequence of naturally occurring, full length CTLA4 (U.S. Patent Nos. 5,434,131, 5,844,095, 5,851,795), or the extracellular domain thereof, which binds a B7, and/or interferes with a B7 from binding their ligands. In particular embodiments, the extracellular domain of wild type CTLA4 begins with methionine at position +1 and ends at aspartic acid at position +124, or the
10 extracellular domain of wild type CTLA4 begins with alanine at position -1 and ends at aspartic acid at position +124. Wild type CTLA4 is a cell surface protein, having an N-terminal extracellular domain, a transmembrane domain, and a C-terminal cytoplasmic domain. The extracellular domain binds to target antigens, such as a B7. In a cell, the naturally occurring, wild type CTLA4 protein is translated as an immature polypeptide,
15 which includes a signal peptide at the N-terminal end. The immature polypeptide undergoes post-translational processing, which includes cleavage and removal of the signal peptide to generate a CTLA4 cleavage product having a newly generated N-terminal end that differs from the N-terminal end in the immature form. One skilled in the art will appreciate that additional post-translational processing may occur, which
20 removes one or more of the amino acids from the newly generated N-terminal end of the CTLA4 cleavage product. The mature form of the CTLA4 molecule includes the extracellular domain of CTLA4, or any portion thereof, which binds to B7.

sub
25 ~~"CTLA4Ig" is a soluble fusion protein comprising an extracellular domain of wild type CTLA4, or a portion thereof that binds a B7, joined to an Ig tail. A particular embodiment comprises the extracellular domain of wild type CTLA4 starting at methionine at position +1 and ending at aspartic acid at position +124; or starting at alanine at position -1 to aspartic acid at position +124; a junction amino acid residue glutamine at position +125; and an immunoglobulin portion encompassing glutamic acid at position +126 through lysine at position +357 (Figure 5).~~
a *and SEQ ID NO. 6*
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As used herein, a "fusion protein" is defined as one or more amino acid sequences joined together using methods well known in the art and as described in U.S. Pat. No. 5,434,131 or 5,637,481. The joined amino acid sequences thereby form one fusion protein.

5 As used herein, "soluble" refers to any molecule, or fragments and derivatives thereof, not bound or attached to a cell i.e. circulating. For example, CTLA4, B7 or CD28 can be made soluble by attaching an immunoglobulin (Ig) moiety to the extracellular domain of CTLA4, B7 or CD28, respectively. Alternatively, a molecule such as CTLA4 can be rendered soluble by removing its transmembrane domain. The soluble molecules used in
10 the methods of the invention may or may not include a signal (or leader) sequence. Typically, the molecules do not include a leader sequence.

As used herein, "soluble CTLA4 molecules" means circulating or non-cell-surface-bound CTLA4 molecules or any functional portion of a CTLA4 molecule that binds B7
15 including, but not limited to: CTLA4Ig fusion proteins, wherein the extracellular domain of CTLA4 is fused to an immunoglobulin (Ig) moiety rendering the fusion molecule soluble, or fragments and derivatives thereof; proteins with the extracellular domain of CTLA4 fused or joined with a portion of a biologically active or chemically active protein such as the papillomavirus E7 gene product (CTLA4-E7), melanoma-associated
20 antigen p97 (CTLA4-p97) or HIV env protein (CTLA4-env gp120), or fragments and derivatives thereof; hybrid (chimeric) fusion proteins such as CD28/CTLA4Ig, or fragments and derivatives thereof; CTLA4 molecules with the transmembrane domain removed to render the protein soluble, or fragments and derivatives thereof. "Soluble CTLA4 molecules" also include fragments, portions or derivatives thereof, and soluble
25 CTLA4 mutant molecules, having CTLA4 binding activity. The soluble CTLA4 molecules used in the methods of the invention may or may not include a signal (or leader) sequence. Typically, the molecules do not include a leader sequence.

As used herein, a "CTLA4 mutant molecule" means wildtype CTLA4 or portions thereof
30 (derivatives or fragments) that have a mutation or multiple mutations (preferably in the extracellular domain of wildtype CTLA4). A CTLA4 mutant molecule has a sequence

that it is similar but not identical to the sequence of wild type CTLA4 molecule, but still binds a B7. The mutations may include one or more amino acid residues substituted with an amino acid having conservative (e.g., substitute a leucine with an isoleucine) or non-conservative (e.g., substitute a glycine with a tryptophan) structure or chemical properties, amino acid deletions, additions, frameshifts, or truncations. Mutant CTLA4 molecules may include a non-CTLA4 molecule therein or attached thereto e.g., the extracellular domain of CTLA4, or portions or fragments thereof, joined to an immunoglobulin constant domain, resulting in the CTLA4Ig molecule (ATCC 68629) or the L104EA29YIg molecule (ATCC PTA-2104) which are copending in U.S. Patent Application Serial Numbers 60/287,576 and 60/214,065, incorporated by reference herein). The mutant molecules may be soluble (i.e., circulating) or bound to a cell surface. Additional CTLA4 mutant molecules include those described in U.S. Patent Application Serial Numbers 09/865,321, 60/214,065 and 60/287,576; and in U.S. Patent Numbers 6,090,914 5,844,095 and 5,773,253. CTLA4 mutant molecules can be made synthetically or recombinantly.

As those skilled-in-the-art will appreciate, mutations in a nucleotide sequence may or may not result in a change in the amino acid sequence. In that regard, certain codons encode the same amino acid. Examples include codons CGT, CGG, CGC, and CGA encoding the amino acid, arginine (R); or codons GAT, and GAC encoding the amino acid, aspartic acid (D). Thus, a protein can be encoded by one or more nucleic acid molecules that differ in their specific nucleotide sequence, but still encode protein molecules having identical sequences. The amino acid coding sequence is as follows:

<u>Amino Acid</u>	<u>Symbol</u>	<u>One Letter Symbol</u>	<u>Codons</u>
Alanine	Ala	A	GCU, GCC, GCA, GCG
Cysteine	Cys	C	UGU, UGC
Aspartic Acid	Asp	D	GAU, GAC
Glutamic Acid	Glu	E	GAA, GAG
Phenylalanine	Phe	F	UUU, UUC
Glycine	Gly	G	GGU, GGC, GGA, GGG

<u>Amino Acid</u>	<u>Symbol</u>	<u>One Letter Symbol</u>	<u>Codons</u>
Histidine	His	H	CAU, CAC
Isoleucine	Ile	I	AUU, AUC, AUA
Lysine	Lys	K	AAA, AAG
Leucine	Leu	L	UUA, UUG, CUU, CUC, CUA, CUG
Methionine	Met	M	AUG
Asparagine	Asn	N	AAU, AAC
Proline	Pro	P	CCU, CCC, CCA, CCG
Glutamine	Gln	Q	CAA, CAG
Arginine	Arg	R	CGU, CGC, CGA, CGG, AGA, AGG
Serine	Ser	S	UCU, UCC, UCA, UCG, AGU, AGC
Threonine	Thr	T	ACU, ACC, ACA, ACG
Valine	Val	V	GUU, GUC, GUA, GUG
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAU, UAC

As used herein "the extracellular domain of CTLA4" is any portion of CTLA4 that recognizes and binds a B7. For example, an extracellular domain of CTLA4 comprises methionine at position +1 to aspartic acid at position +124 (Figure 5). ^{and SEQ ID NO. 6} Alternatively, an extracellular domain of CTLA4 comprises alanine at position -1 to aspartic acid at position +124 (Figure 5). ^{and SEQ ID NO. 6} The extracellular domain includes fragments or derivatives of CTLA4 that bind a B7.

As used herein, "lymphocyte" refers to mononuclear cells that mediate humoral- or cell-mediated immunity. Major subsets of lymphocytes include B and T cells.

As used herein, "immune system diseases" refer to autoimmune, immunoproliferative disorders and graft-related disorders including, but not limited to: graft-versus-host disease (GVHD) (e.g., such as may result from bone marrow transplantation, or in the induction of tolerance); immune disorders associated with graft transplantation rejection (e.g. chronic rejection, tissue or cell allo- or xenografts including solid organs, skin, islets,

muscles, hepatocytes, neurons, etc.); T cell lymphoma; psoriasis; T cell acute lymphoblastic leukemia; testicular angiocentric T cell lymphoma; benign lymphocytic angiitis; and autoimmune diseases such as lupus (e.g. lupus erythematosus, lupus nephritis), Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, diabetes (e.g. insulin dependent diabetes mellitus, non-insulin dependent diabetes), good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic diseases (e.g. rheumatoid arthritis), polymyositis, scleroderma, and mixed connective tissue disease.

As used herein, "subject" means any living organism to which the agents can be administered in order to regulate an immune response. Subjects may include, but are not limited to, humans, monkeys, mice, rats, cats, dogs, hamsters, any transgenic animals, any allograft recipients, any xenograft recipients or any graft recipients.

As used herein, "administer" means to provide an agent to a subject by any convenient method, including, but not limited to, oral administration, inhalation administration, intravenous administration, intraperitoneal administration, subcutaneous administration, intramuscular administration, administration by suppositories or topical contact, or administration by slow release devices such as vesicles or capsules.

As used herein, "gene therapy" is a process to treat a disease by genetic manipulation so that a sequence of nucleic acid is transferred into a cell, the cell then expressing any genetic product encoded by the nucleic acid. For example, as is well known by those skilled in the art, nucleic acid transfer may be performed by inserting an expression vector containing the nucleic acid of interest into cells *ex vivo* or *in vitro* by a variety of methods including, for example, calcium phosphate precipitation, diethylaminoethyl dextran, polyethylene glycol (PEG), electroporation, direct injection, lipofection or viral infection (63, 64, 65). Alternatively, nucleic acid sequences of interest may be transferred into a cell *in vivo* in a variety of vectors and by a variety of methods

including, for example, direct administration of the nucleic acid into a subject (66), or insertion of the nucleic acid into a viral vector and infection of the subject with the virus (67-70). Other methods used for *in vivo* transfer include encapsulation of the nucleic acid into liposomes, and direct transfer of the liposomes, or liposomes combined with a hemagglutinating Sendai virus, to a subject (71). The transfected or infected cells express the protein products encoded by the nucleic acid in order to ameliorate a disease or the symptoms of a disease. The expressed protein products may be secreted or remain in the transfected or infected cells.

10 As used herein, "pharmaceutically acceptable carrier" means any material that may be combined with the agents in order to administer the agents to a subject in any form. For example, a carrier includes any material that will maintain the agents' effective activity when administered to a subject and that is non-reactive with a subject's immune system. Potential carriers may include, but are not limited to, any solvents, media, suspensions, emulsions or other excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acids, stearate salts, talcum, oils, gums, glycols, flavorings, preservatives or color additives, etc. Potential carrier forms may include sterile solutions, aerosols, liposomes, vesicles, suppositories, pills, tablets or capsules.

20 In order that the invention herein described may be more fully understood, the following description is set forth.

METHODS OF THE INVENTION

25 This invention provides methods for regulating cell-mediated immune responses by blocking interactions between receptors on lymphocytes such as CTLA4, CD28, B7, CD40 or CD154, with their ligands and also by blocking LFA-1-mediated interactions. The method involves: blocking a first lymphocytic signal by contacting a molecule such as CD28, CTLA4 or B7 on a lymphocyte with a first agent; blocking a second lymphocytic signal by contacting a molecule such as CD40 or CD154 on a lymphocyte with a second agent; and blocking adhesion molecule mediated interactions with its

ligands by contacting an adhesion molecule (e.g. LFA-1) or the ligands of the adhesion molecule with a third agent.

This invention provides methods for treating an immune system disease by blocking interactions between receptors on lymphocytes such as CTLA4, CD28, B7, CD40 or CD154, with their ligands and also by blocking LFA-1-mediated interactions. The method comprises administering at least three agents: a first agent which blocks a CD28/CTLA4/B7-mediated signal by binding CD28, CTLA4 or B7; a second agent which blocks a CD40/CD154-mediated signal by binding either CD40 or CD154; and a third agent which blocks an adhesion molecule-mediated interaction by binding to LFA-1, ICAM-1, ICAM-2, ICAM-3, α -actinin, filamin or cytohesin-1. Blockage by a combination of any of the three agents therapeutically treating an immune system related disease.

15 The first agent preferably acts by interfering with the interaction between a receptor on a lymphocyte (e.g., CD28 and/or CTLA4) and its ligand (e.g., B7-1 and/or B7-2). Examples of the first agent include, but are not limited to, molecules such as an antibody (or portion or derivative thereof) that recognizes and binds to the receptor or the ligand; a soluble form (or portion or derivative thereof) of the receptor or the ligand such as soluble CTLA4; a peptide fragment or other small molecule designed to interfere with the lymphocytic signal through the receptor/ligand mediated interaction. In a preferred embodiment, the first agent is a soluble CTLA4 molecule, such as CTLA4Ig (ATCC 68629) or L104EA29YIg (ATCC PTA2104), a soluble CD28 molecule such as CD28Ig (ATCC 68628), a soluble B7 molecule such as B7Ig (ATCC 68627), an anti-B7 monoclonal antibody (e.g. ATCC HB-253, ATCC CRL-2223, ATCC CRL-2226, ATCC HB-301, ATCC HB-11341 and monoclonal antibodies as described in references 80-81), an anti-CTLA4 monoclonal antibody (e.g. ATCC HB-304, and monoclonal antibodies as described in references 82-83) and/or an anti-CD28 monoclonal antibody (e.g. ATCC HB 11944 and mAb 9.3 as described in reference 79).

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5 The second agent acts by interfering with the interaction between a second receptor on a lymphocyte (e.g., CD154) and its ligand (e.g., CD40). Examples of the second agent include, but are not limited to, molecules such as an antibody (or portion or derivative thereof) that recognize and bind the second receptor or the ligand such as an anti-CD154 monoclonal antibody; a soluble form (or portion or derivative thereof) of the receptor or the ligand; a peptide fragment or other small molecule designed to interfere with the lymphocytic signal through the second receptor/ligand mediated interaction. In a preferred embodiment, the second agent is an anti-CD154 (e.g. MR1 as described in reference 56, ATCC HB-10916, ATCC HB-12055 and ATCC HB-12056) and/or anti-
10 CD40 monoclonal antibody (e.g. ATCC HB-9110).

15 The third agent interferes with adhesion molecule (e.g. LFA-1) interactions with its ligands. Examples of adhesion molecules and ligands include, but are not limited to, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18), ICAM (1, 2 and 3), VLA-1, CD44, CD62 (E, L and P), CD106, fibrinogen, α -actinin, filamin and cytohesin-1. LFA-1 ligands such as ICAM-1, ICAM-2, ICAM-3, α -actinin, filamin and cytohesin-1, etc., can be located on another cell or in the extracellular matrix. Examples of the third agent include, but are not limited to: molecules such as an antibody (or portion or derivative thereof) that recognizes and binds adhesion molecules or its ligands;
20 a soluble form (or portion or derivative thereof) of the adhesion molecule or its ligand; a peptide fragment or other small molecule designed to interfere with the adhesion molecule/ligand interaction. In a preferred embodiment, the third agent is an anti-LFA-1 (e.g. ATCC HB-9579, and ATCC TIB-213), anti-ICAM-1 (e.g. ATCC CRL-1878 and ATCC HB-233), anti-ICAM-2, anti-ICAM-3, anti- α -actinin (e.g. ATCC CRL-2252),
25 anti-filamin, anti-cytohesin-1, anti-CD11a (e.g. M17/5.2 ATCC TIB-237, ATCC HB-202, ATCC HB-244; and ATCC TIB-217) and/or anti-CD18 (ATCC HB-203, ATCC HB-226 and ATCC TIB-218) monoclonal antibody.

30 A preferred embodiment of the invention regulates a cell-mediated immune response by blocking at least three cellular pathways: the CD28/CTLA4/B7-mediated pathway with a first agent, CTLA4Ig; the CD40/CD154 mediated pathway with a second agent, anti-

CD154 monoclonal antibody MR1; and an adhesion molecule mediated pathway with a third agent, anti-LFA-1 monoclonal antibody M17/5.2.

Further aspects of the invention encompass one or more agents targeted to one or more of the pathways described above to regulate an immune response. For example, the first and second agents may interfere with the CD28/CTLA4/B7 pathway (e.g. CTLA4Ig plus an anti-B7 monoclonal antibody), and the third agent may interfere with an adhesion molecule mediated pathway (e.g. anti-LFA-1 monoclonal antibody). Alternatively, all three agents may interfere with one pathway (e.g. CTLA4Ig, an anti-B7-1 monoclonal antibody plus an anti-B7-2 monoclonal antibody).

The invention includes pharmaceutical compositions for use in the treatment of immune system diseases comprising pharmaceutically effective amounts of the three agents described above. Accordingly, a cell-mediated immune response to antigen challenge can be regulated by application of a combination of at least three agents to a subject in order to disrupt at least three cellular contacts. Subjects may include, but are not limited to, humans, monkeys, mice, rats, cats, dogs, hamsters, etc. The compositions may additionally include other therapeutic agents, including, but not limited to, drug toxins, enzymes, antibodies (or portions or derivative thereof), or conjugates.

The administration of the agents may occur at the same time or at different times. For example, the agents can be administered in a specified order and can be applied concurrently or sequentially in a time-dependant application or any combination thereof. The agents may be administered sequentially or concurrently and in any order. A subject may be treated with the agents before, during, or after (or any combination thereof) an immune response to antigen presentation depending on the dosage and mode of application.

Dosage of the agents is dependant upon many factors including, but not limited to, the type of subject (i.e. the species), the agent used (e.g. CTLA4Ig or L104EA29YIg), location of the antigenic challenge, the type of tissue affected, the type of immune system

disease being treated, the severity of the disease, a subject's health and response to the treatment with the agents. Accordingly, dosages of the agents can vary depending on each subject, agent and the mode of administration. For example, soluble CTLA4 molecules such as L104EA29YIg (included in Figure 6; and SEQs: 8 and 9 as encoded by DNA deposited with ATCC accession number PTA-2104; and as described in U.S. Patent Application Serial Numbers 09/579,927, 60/287,576 and 60/214,065, incorporated by reference herein), may be administered in an amount between 0.1 to 20.0 mg/kg weight of a human subject/day, preferably between 0.5 to 10.0 mg/kg/day.

- Administration of the agents may be performed in many permissible ways including, but not limited to: injection (e.g. intravenous, intraperitoneal, intramuscular, etc.), oral administration, inhalation, topical contact, gene therapy, administration by a mechanical release device such as a pump, administration of slow release devices such as vesicles or capsules, or suppositories. Depending on the means of administration, the agents may be compounded with pharmaceutically acceptable carriers for convenient application and effective use of the agents.

- The pharmaceutical compositions also preferably include suitable carriers and adjuvants which include any material which when combined with the molecule of the invention (e.g., a soluble CTLA4 mutant molecule, such as, L104EA29Y or L104E) retains the molecule's activity and is non-reactive with the subject's immune system. Examples of suitable carriers and adjuvants include, but are not limited to, human serum albumin; ion exchangers; alumina; lecithin; buffer substances, such as phosphates; glycine; sorbic acid; potassium sorbate; and salts or electrolytes, such as protamine sulfate. Other examples include any of the standard pharmaceutical carriers such as a phosphate buffered saline solution; water; emulsions, such as oil/water emulsion; and various types of wetting agents. Other carriers may also include sterile solutions; tablets, including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

Compositions comprising such carriers are formulated by well known conventional methods. Such compositions may also be formulated within various lipid compositions, such as, for example, liposomes as well as in various polymeric compositions, such as polymer microspheres.

5

Administration of the agents in any form to regulate a cell-mediated immune response in turn can affect the development of immune system diseases such as immunoproliferative diseases or autoimmune diseases/disorders of an immune system. Examples of immune system diseases include, but are not limited to, graft versus host disease (GVHD), psoriasis, immune disorders associated with graft transplant rejection (e.g. allograft transplant rejection), T cell lymphoma, T cell acute lymphoblastic leukemia, testicular angiocentric T cell lymphoma, benign lymphocytic angiitis, lupus (e.g. lupus erythematosus, lupus nephritis), Hashimoto's thyroiditis, primary myxedema, Graves' disease, pernicious anemia, autoimmune atrophic gastritis, Addison's disease, diabetes (e.g. insulin dependent diabetes mellitus, type I diabetes mellitus), good pasture's syndrome, myasthenia gravis, pemphigus, Crohn's disease, sympathetic ophthalmia, autoimmune uveitis, multiple sclerosis, autoimmune hemolytic anemia, idiopathic thrombocytopenia, primary biliary cirrhosis, chronic action hepatitis, ulceratis colitis, Sjogren's syndrome, rheumatic diseases (e.g. rheumatoid arthritis), polymyositis, scleroderma, and mixed connective tissue disease.

20

The present invention further provides methods for treating immune system diseases in a subject. Examples of immune system diseases are as described *supra*. In one example, the invention provides a method for treating an autoimmune disorder. Many autoimmune disorders result from inappropriate activation of T lymphocytes that are reactive against autoantigens, and which promote the production of cytokines and autoantibodies that are involved in the pathology of the disease. Administration of the three agents of the invention to a subject suffering from or susceptible to an autoimmune disorder may prevent the activation of autoreactive T cells and may reduce or eliminate disease symptoms.

30

In a preferred embodiment of the invention, administration of a combination of at least three agents, CTLA4Ig, anti-CD154 monoclonal antibody and anti-LFA-1 monoclonal antibody, regulates an immune response as monitored by allograft transplant survival assays.

5

The present invention further provides a method for inhibiting allograft transplant rejections by a subject. Typically, in transplants, rejection of the graft is initiated through its recognition as foreign by T cells, followed by an immune response that destroys the graft. The three agents of this invention, by inhibiting the interaction of cell surface molecules with their ligands, block immune responses subsequent to allograft transplantation. Examples of immune responses affected by administration of the agents include inhibition of T lymphocyte proliferation and/or cytokine secretion that may result in reduced tissue destruction and induction of antigen-specific T cell unresponsiveness in long-term graft acceptance without the need for generalized immunosuppression. The invention thus enhances allograft survival.

Furthermore, the agents of the invention can be administered with other pharmaceuticals including, but not limited to, other drugs. For example, it may be used in combination with a calcineurin inhibitor, e.g. cyclosporin A or FK506; an immunosuppressive macrolide, e.g. rapamycin or a derivative thereof; e.g. 40-O-(2-hydroxy)ethyl-rapamycin, a lymphocyte homing agent, e.g. FTY720 or an analog thereof; corticosteroids; cyclophosphamide; azathioprene; methotrexate; leflunomide or an analog thereof; mizoribine; mycophenolic acid; mycophenolate mofetil; 15-deoxyspergualine or an analog thereof; immunosuppressive monoclonal antibodies (or portions or derivative thereof), e.g., monoclonal antibodies to leukocyte receptors (or portions or derivative thereof), e.g., MHC, CD2, CD3, CD4, CD 11a/CD18, CD7, CD25, CD 27, B7, CD40, CD45, CD58, CD 137, ICOS, CD150 (SLAM), OX40, 4-1BB or their ligands; or other immunomodulatory compounds, e.g. Selectin antagonists and VLA-4 antagonists.

30 Where the soluble CTLA4 mutant molecules of the invention are administered in conjunction with other immunosuppressive / immunomodulatory or anti-inflammatory

therapy, e.g. as hereinabove specified, dosages of the co-administered immunosuppressant, immunomodulatory or anti-inflammatory compound will of course vary depending on the type of co-drug employed, e.g. whether it is a steroid or a cyclosporine, on the specific drug employed, on the condition being treated and so forth.

5

In accordance with the foregoing, the present invention provides in a yet further aspect methods as defined above comprising co-administration, e.g. concomitantly or in sequence, of a therapeutically effective amount of the molecules of the invention, in free form or in pharmaceutically acceptable salt form, and a second drug substance, said
10 second drug substance being an immunosuppressant, immunomodulatory or anti-inflammatory drug, e.g. as indicated above. Further provided are therapeutic combinations, e.g. a kit, e.g. for use in any method as defined above, comprising the molecules of the invention in free form or in pharmaceutically acceptable salt form, to be used concomitantly or in sequence with at least one pharmaceutical composition
15 comprising an immunosuppressant, immunomodulatory or anti-inflammatory drug.

Gene therapy is currently being used to treat a variety of immune system diseases such as immunoproliferative diseases (e.g. cancers) or autoimmune diseases (e.g. diabetes). The agents of this invention may be administered to a subject by gene therapy in order to
20 regulate transplant rejection and treat immune system diseases. Gene therapy methods include, but are not limited to, *in vivo* methods directly delivering the genes into a subject for *in situ* transfer into a subject's cells, or *ex vivo* or *in vitro* methods transferring the genes into cells in culture and then transplanting the cultured cells into a subject. Once the gene of interest, encoding one or more of the therapeutic agents, are inserted into cells
25 in a subject, the gene product is expressed and used to ameliorate the disease or symptoms of the disease.

In vivo, potential methods for inserting the gene of interest directly into a cell include the usage of recombinant viral vectors such as simian virus 40, adenoviruses, human
30 immunodeficiency virus-1, or other viruses. Methods such as microinjection of the gene of interest directly into cells or other transfer procedures may also be employed.

Ex vivo or *in vitro*, potential methods for inserting the gene of interest into cells include transfer procedures such as calcium phosphate mediated transfection, lipid mediated transfection, exposure of the cells to high voltage electric currents to permeabilize the cells (electroporation), microinjection, or encapsulation of the gene of interest into erythrocyte ghosts for transfer into cells.

Gene therapy may be used to treat or augment treatment of various diseases. For example, in the present invention, the DNA encoding one or more of the agents such as soluble CTLA4Ig or soluble gp39 may be inserted into a retroviral vector and packaged into a virus particle. The virus particle is used to infect a subject's cells, transferring the genes of interest into the subject. Once the genes are transferred into a subject, the gene product is expressed in a sufficient amount to regulate transplant rejection and treat immune system diseases.

The present invention provides methods for regulating cell-mediated immune responses and treating diseases in subjects by disrupting the interaction between cell surface molecules with various ligands, thus inducing improved regulation of immune responses and treatment of immune system diseases. Specific interactions that may be disrupted include, but are not limited to, the CTLA4/B7, CD28/B7, CD154/CD40 and LFA-1/ICAM pathways. Disruption of three or more interactions leads to enhanced regulation of immune responses, enhanced transplant survival, and enhanced regulation of immunoproliferation.

The invention also provides kits for use in the methods of the invention. Kits may include the three agents of the invention in free form or combined with a pharmaceutically acceptable carrier. Additionally, the kits may also include one or more immunosuppressive agents in conjunction with the three agents of the invention. Potential immunosuppressive agents include, but are not limited to, corticosteroids, nonsteroidal antiinflammatory drugs (e.g. Cox-2 inhibitors), cyclosporin prednisone, azathioprine, methotrexate, TNF α blockers or antagonists, infliximab, any biological

agent targeting an inflammatory cytokine, hydroxychloroquine, sulphasalazopyrine, gold salts, etanercept, and anakinra.

In addition to the molecules identified herein for the methods of the invention, other
5 molecules (i.e. ligands) can be identified using standard techniques such as binding
assays. For example, any of the molecules of the invention (e.g. CTLA4, CD28, B7,
CD154, CD40, LFA-1, ICAM-1, ICAM-2, ICAM-3, α -actinin, filamin or cytohesin-1
interaction) can be used to screen for ligand molecules including libraries of small
molecules in any of a variety of screening techniques. The molecules of the invention
10 employed in such screening may be free in solution, affixed to a solid support, or borne
on a cell surface. The formation of binding complexes, between any of the molecules of
the invention and the agent being tested, may be measured (e.g. published PCT
application WO84/03564; Price, M.R., et al. 1986. Br. J. Cancer 54:393 (88); Gallegher,
G., et al, 1993. Tumour Immunobiology, pages 63-79, Oxford University Press Inc., New
15 York (89)).

Another screening technique involves high throughput screenings of molecules having
suitable binding affinity to the molecule of the invention as described in published PCT
application WO84/03564. In this method, as applied to the molecules of the invention,
20 large number of different small test molecules can be synthesized on a solid substrate,
such as plastic pins or some other surface. The test molecules can be reacted with the
molecules of the invention, and washed. Bound molecules of the invention can be then
detected by methods well known in the art. Purified molecules of the invention can be
directly coated on to plates for use in the screening assay. Alternatively, non-neutralizing
25 antibodies (or portions or derivative thereof) can be used to capture the molecule of the
invention and immobilize it on a solid support.

Another competitive screening assay involves the use of neutralizing antibodies (capable
of binding the molecules of the invention) to specifically compete with a test molecule
30 for binding to the molecule of the invention. In this manner, the antibodies can be used to

detect the presence of any protein/peptide molecule which shares one or more antigenic determinants with the molecule of the invention.

As will be apparent to those skilled in the art to which the invention pertains, the present invention may be embodied in forms other than those specifically disclosed herein without departing from the spirit or essential characteristics of the invention. The particular embodiments of the invention described herein, are, therefore, to be considered as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the description that follows.

EXAMPLE 1: MURINE TAIL SKIN TRANSPLANTS

Materials and Methods

Mice. Adult male 6-8 week old C57BL/6 and BALB/c mice were purchased from Harlan (Indianapolis, Indiana). C57BL/6 mice were transplant donors and BALB/c mice were recipients.

Reagents. Murine CTLA4Ig (Linsley (13) and Wallace (87)) and monoclonal antibodies MR1 (hamster anti-murine CD40L/CD154) (56) and M17/5.2 (anti-murine LFA-1/CD11a) have been described previously and were purified from culture supernatants prior to use (62).

Murine Tail Skin Transplants. In order to examine skin graft rejection rates in mice, tail skin grafts were studied. Briefly, donor tail skin is removed aseptically from the euthanized donor mice. The tail skin of the recipient is surgically prepped with alcohol and an approximately 1x0.5 cm area of recipient skin is removed from the underside of the tail to form a grafting bed. A skin graft from the donor of equal size to the bed is then placed on the bed and covered with a protective glass tube which is fastened to the tail by

surgical tape for 2-3 days at which time the tube and tape is removed. Grafts are examined daily for necrosis and are deemed rejected when > 75% necrotic.

5 The tail skin transplant was performed on day 0. The three agents, CTLA4Ig, MR1 (hamster anti-murine CD40L/CD154) and M17/5.2 (anti-murine LFA-1/CD11a), were administered intraperitoneally, individually or in combinations of two or three agents to the mice. CTLA4Ig was administered in 200 µg dosages on days 0, 2 and 4 post-transplantation. MR1 was administered in 250 µg dosages on days 0, 2 and 4 post-transplantation. Anti-LFA-1 monoclonal antibody was administered in 200 µg dosages
10 on days 0, 1, 2, 3, 4, 5, 6, 14 and 21 post-transplantation.

Results

15 *Anti-LFA-1 monoclonal antibody augments lymphocyte interaction blockade induced graft survival following murine skin transplantation.*

Blockade of the LFA-1/ICAM-1 interaction has been previously shown to extend allogeneic skin graft survival in murine models of transplantation. In these models, long-term graft survival is achieved only when a combination of antibodies directed at both
20 LFA-1 and ICAM-1 were administered together. Again, previous studies have demonstrated that soluble CTLA4Ig and anti-CD154 monoclonal antibody MR1, when combined, provide long-term graft survival of allogeneic skin transplants in certain donor/recipient strain combinations. In the BALB/c->C57BL/6 combination, soluble CTLA4Ig and anti-CD154 antibody MR1 when administered alone do not extend graft
25 survival and when administered in combination do not provide long-term graft survival.

To evaluate the effect of combining anti-LFA-1 monoclonal antibody administration with either soluble CTLA4Ig or anti-CD154 antibody administration, BALB/c donor skin was transplanted onto the tail of C57BL/6 recipients treated with anti-LFA-1 monoclonal
30 antibody M17/5.2, soluble CTLA4Ig, and anti-CD154 monoclonal antibody MR1, or combinations of the three agents. Graft survival was monitored daily and the results are

shown in Figure 1. The data demonstrate that administration of anti-LFA-1 along with soluble CTLA4Ig profoundly enhances graft survival when compared to either agent alone. Median graft survival of anti-LFA-1+CTLA4Ig treated mice (55.0 days) was more than either anti-LFA-1 (22.0 days) or soluble CTLA4Ig (21.0 days) alone. Likewise, the data show that combined administration of anti-LFA-1 and anti-CD154 antibody MR1 greatly enhanced graft survival from a median graft survival of 22.0 days and 14.0 days for either anti-LFA-1 or anti-CD154 alone to 34.0 days when administered together. As previously reported, administration of soluble CTLA4Ig and MR1 together does not lead to long-term graft survival (MST=29.0 days) in this skin transplant model; however the addition of anti-LFA-1 monoclonal antibody to this regimen increased median graft survival to 65.0 days. Thus the triple therapy was more successful than double therapy.

EXAMPLE 2: MURINE NEONATAL HEART TO EAR TRANSPLANTS.

Materials and Methods

Mice. Adult male 6-8 week old C57BL/6 and BALB/c mice were purchased from Harlan (Indianapolis, Indiana). Neonatal C57BL/6 heart donors were bred in our facility. C57BL/6 mice were donors and BALB/c mice were recipients.

Reagents. Murine CTLA4-Ig (Linsley (13) and Wallace (84)) and monoclonal antibodies MR1 (hamster anti-murine CD40L/CD154) (56) and M17/5.2 (anti-murine LFA-1/CD11a) have been described previously and were purified from culture supernatants prior to use (62).

Murine Neonatal Heart to Ear Transplants. Cardiac transplants were performed as described by Fulmer *et al* (57). Neonatal hearts are obtained from newborn mice less than 48 hours after birth. The heart is transplanted into recipient mice, which are prepared under anesthesia by making a small incision at the base of the ear pinna and forming a pocket by gently lifting the skin away from the ear. The incision permits the insertion of the donor heart subcutaneously into the pocket. Post-operatively, the cardiac tissue does

not begin to beat until 3 to 5 days after transplantation until vascular connections are established. The pulsations of the heart can often be observed under magnification or by measuring contractile activity with an appropriate ECG monitoring device. The mice are observed daily and contractile activity of the transplanted graft monitored daily. The
5 time of graft rejection is defined as the day after transplantation on which contractile activity ceases.

The three agents, CTLA4-Ig, MR1 (hamster anti-murine CD40L/CD154) and M17/5.2 (anti-murine LFA-1/CD11a), were administered intraperitoneally, independently or in
10 combinations of two or three agents to the mice. CTLA4-Ig was administered in 200 µg dosages on days 0, 2 and 4 post-transplantation. MR1 was administered in 250 µg dosages on days 0, 2 and 4 post-transplantation. Anti-LFA-1 monoclonal antibody was administered in 200 µg dosages on days 0, 2, 4 and 6 post-transplantation.

15 On the days 10, 17 and 40 as indicated in Figures 3 and 4, the mice were euthanized and the grafts were removed and placed in formalin for histopathology. To examine inflammatory infiltration and myocardium damage, hematoxylin and eosin (H&E) staining on cut sections was performed. The results of the myocardium damage analysis are shown in Figure 3 with the figure indicating the percent cardiac tissue remaining in
20 the graft for each group i.e. higher scores indicate a greater percentage of cardiac tissue present and, accordingly, less tissue damage. The results of the inflammatory infiltration are shown in Figure 4 with the lower scores indicating less inflammation present in the graft for the various treatment groups.

25 Results

Anti-LFA-1 monoclonal antibody augments lymphocyte interaction blockade induced graft survival following murine cardiac transplantation.

30 Blockade of the LFA-1/ICAM-1 interaction has been previously shown to extend allogeneic cardiac graft survival in murine models of transplantation. In these models,

long-term graft survival is achieved only when a combination of antibodies directed at both LFA-1 and ICAM-1 were administered together. Likewise, previous studies have demonstrated that soluble CTLA4-Ig and anti-CD154 antibody when combined, provide long-term graft survival of allogeneic heart transplants. Alone, each agent extends graft survival although significantly less than when administered together.

To evaluate the effect of combining anti-LFA-1 administration with either soluble CTLA4-Ig or anti-CD154 administration, neonatal C57BL/6 hearts were subcutaneously implanted into the ear pinna of adult BALB/c recipients treated with anti-LFA-1 monoclonal antibody, soluble CTLA4-Ig, anti-CD154 monoclonal antibody, or combinations of the three agents. Contractile activity of these hearts were monitored daily by ECG and the heart graft survival results are shown in Figure 2. The data demonstrate that administration of anti-LFA-1 along with soluble CTLA4-Ig profoundly enhances graft survival when compared to either agent alone. Median graft survival of anti-LFA-1+CTLA4-Ig treated mice (57 days) was more than double either anti-LFA-1 (18 days) or soluble CTLA4-Ig (20 days) alone ($p<0.0003$). Likewise, the data show that combined administration of anti-LFA-1 and anti-CD154 greatly enhanced graft survival from a median graft survival of 18.0 days and 33.0 days for either anti-LFA-1 or anti-CD154 alone to 67.5 days when administered together. As previously reported, administration of soluble CTLA4-Ig and anti-CD40L leads to very long-term graft survival (MST>80 days).

The data results further indicate that the groups that received multiple therapies (CTLA4Ig, MR1 and anti-LFA-1 either in double or triple combinations), retained a higher percentage of myocardial tissue (Figure 3) and had less inflammation (Figure 4) than the other therapy groups.

EXAMPLE 3: CONSTRUCTION OF HUMAN CTLA4Ig AND L104EA29YIg

This example provides a description of the methods used to generate the nucleotide sequences encoding the human soluble CTLA4 molecules e.g. CTLA4Ig, L104EA29YIg.

A nucleotide sequence encoding CTLA4Ig was first generated as described *infra*, then a single-site mutant L104EIg was derived from the CTLA4Ig sequence and tested for binding kinetics for CD80 and/or CD86. The L104EIg nucleotide sequence was used as a template to generate the double-site mutant CTLA4 sequence, L104EA29YIg, which
5 was tested for binding kinetics for CD80 and/or CD86.

Construction of CTLA4Ig

A genetic construct encoding CTLA4Ig comprising the extracellular domain of CTLA4 and
10 an IgCgamma1 domain was constructed as described in U.S. Patents 5,844,095 and 5,851,795, the contents of which are incorporated by reference herein. The extracellular domain of the CTLA4 gene was cloned by PCR using synthetic oligonucleotides corresponding to the published sequence as described by Dariavach et al. (72).

15 Because a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N-terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (73) in two steps using overlapping oligonucleotides. For the first step, the oligonucleotide, CTCAGTCTGGTCCTTGCACTCCTGTTTCCAAGCATGGCGAGCA
TGGCAATGCACGTGGCCCAGCC (which encoded the C terminal 15 amino acids
20 from the oncostatin M signal peptide fused to the N terminal 7 amino acids of CTLA4)

was used as forward primer, and TTTGGGCTCCTGATCAGAATCTGGGCACGGTTG
(encoding amino acid residues 119-125 of the amino acid sequence encoding CTLA4
receptor and containing a Bcl I restriction enzyme site) as reverse primer. The template
for this step was cDNA synthesized from 1 micro g of total RNA from H38 cells (an
25 HTLV II infected T-cell leukemic cell line provided by Drs. Salahudin and Gallo, NCI, Bethesda, MD). A portion of the PCR product from the first step was reamplified, using an overlapping forward primer, encoding the N terminal portion of the oncostatin M signal peptide and containing a Hind III restriction endonuclease site,
CTAGCCACTGAAGCTTCACCAATGGGTGTACTGCTCACACAGAGGACGCTGC
30 TCAGTCTGGTCCTTGCACTC and the same reverse primer. The product of the PCR
reaction was digested with Hind III and Bcl I and ligated together with a Bcl I/Xba I

a cleaved cDNA fragment encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of Ig^{gamma}C1 into the Hind III/Xba I cleaved expression vector, CDM8 or Hind III/Xba I cleaved expression vector pILN (also known as π LN).

~~Copy~~ DNA encoding the amino acid sequence corresponding to CTLA4Ig has been deposited with the ATCC under the Budapest Treaty on May 31, 1991, and has been accorded ATCC accession number 68629.

CTLA4Ig Codon Based Mutagenesis to generate double mutants:

10 A mutagenesis and screening strategy was developed to identify mutant CTLA4Ig molecules that had slower rates of dissociation ("off" rates) from CD80 and/or CD86 molecules. Single-site mutant nucleotide sequences were generated using CTLA4Ig (U.S. Patent Numbers: 5,844,095; 5,851,795; and 5,885,796; ATCC Accession No. 68629) as a template. Mutagenic oligonucleotide PCR primers were designed for random
15 mutagenesis of a specific cDNA codon by allowing any base at positions 1 and 2 of the codon, but only guanine or thymine at position 3 (XXG/T; also known as NNG/T). In this manner, a specific codon encoding an amino acid could be randomly mutated to code for each of the 20 amino acids. In that regard, XXG/T mutagenesis yields 32 potential codons encoding each of the 20 amino acids. PCR products encoding mutations in close
20 proximity to -M97-G107 of CTLA4Ig (see Figure 5), were digested with SacI/XbaI and subcloned into similarly cut CTLA4Ig π LN expression vector. This method was used to generate the single-site CTLA4 mutant molecule L104EIg.

a For mutagenesis in proximity to S25-R33 of CTLA4Ig, a silent NheI restriction site was
25 first introduced 5' to this loop, by PCR primer-directed mutagenesis. PCR products were digested with NheI/XbaI and subcloned into similarly cut CTLA4Ig or L104EIg expression vectors. This method was used to generate the double-site CTLA4 mutant molecule L104EA29YIg (Figure 6). In particular, the nucleic acid molecule encoding the
a single-site CTLA4 mutant molecule, L104EIg, was used as a template to generate the

double-site CTLA4 mutant molecule, L104EA29YIg. The sequence of L104EA29YIg is shown in Figure 6 and includes an N-terminal leader sequence.

The following provides a description of the screening methods used to identify the single- and double-site mutant CTLA4 polypeptides, expressed from the constructs described *supra*, that exhibited a higher binding avidity for CD80 and CD86 antigens, compared to non-mutated CTLA4Ig molecules.

Current *in vitro* and *in vivo* studies indicate that CTLA4Ig by itself is unable to completely block the priming of antigen specific activated T cells. *In vitro* studies with CTLA4Ig and either monoclonal antibody specific for CD80 or CD86 measuring inhibition of T cell proliferation indicate that anti-CD80 monoclonal antibody did not augment CTLA4Ig inhibition. However, anti-CD86 monoclonal antibody did augment the inhibition, indicating that CTLA4Ig was not as effective at blocking CD86 interactions. These data support earlier findings by Linsley et al. (74) showing inhibition of CD80-mediated cellular responses required approximately 100 fold lower CTLA4Ig concentrations than for CD86-mediated responses. Based on these findings, it was surmised that soluble CTLA4 mutant molecules having a higher avidity for CD86 than wild type CTLA4 should be better able to block the priming of antigen specific activated cells than CTLA4Ig.

To this end, the soluble CTLA4 mutant molecules described above were screened using a novel screening procedure to identify several mutations in the extracellular domain of CTLA4 that improve binding avidity for CD80 and CD86. This screening strategy provided an effective method to directly identify mutants with apparently slower "off" rates without the need for protein purification or quantitation since "off" rate determination is concentration independent as described by O'Shannessy et al (75).

COS cells were transfected with individual miniprep purified plasmid DNA and propagated for several days. Three day conditioned culture media was applied to BIAcore biosensor chips (Pharmacia Biotech AB, Uppsala, Sweden) coated with soluble

CD80Ig or CD86Ig. The specific binding and dissociation of mutant proteins was measured by surface plasmon resonance (75). All experiments were run on BIAcore™ or BIAcore™ 2000 biosensors at 25°C. Ligands were immobilized on research grade NCM5 sensor chips (Pharmacia) using standard N-ethyl-N'-(dimethylaminopropyl) carbodiimidN-hydroxysuccinimide coupling (76, 77).

Screening Method

COS cells grown in 24 well tissue culture plates were transiently transfected with DNA encoding mutant CTLA4Ig. Culture media containing secreted soluble mutant CTLA4Ig was collected 3 days later.

Conditioned COS cell culture media was allowed to flow over BIAcore biosensor chips derivatized with CD86Ig or CD80Ig as described in Greene et al. (78), and mutant molecules were identified with "off" rates slower than that observed for wild type CTLA4Ig. The cDNAs corresponding to selected media samples were sequenced and DNA was prepared to perform larger scale COS cell transient transfection, from which mutant CTLA4Ig protein was prepared following protein A purification of culture media.

BIAcore analysis conditions and equilibrium binding data analysis were performed as described in Greene et al. (78).

BIAcore Data Analysis

Sensorgram baselines were normalized to zero response units (RU) prior to analysis. Samples were run over mock-derivatized flow cells to determine background response unit (RU) values due to bulk refractive index differences between solutions. Equilibrium dissociation constants (K_d) were calculated from plots of R_{eq} versus C , where R_{eq} is the steady-state response minus the response on a mock-derivatized chip, and C is the molar concentration of analyte. Binding curves were analyzed using commercial nonlinear curve-fitting software (Prism, GraphPAD Software).

Experimental data were first fit to a model for a single ligand binding to a single receptor (1-site model, i.e., a simple langmuir system, $A+B \leftrightarrow AB$), and equilibrium association constants ($K_d = [A] \cdot [B] / [AB]$) were calculated from the equation $R = R_{\max} \cdot C / (K_d + C)$.

- 5 Subsequently, data were fit to the simplest two-site model of ligand binding (i.e., to a receptor having two non-interacting independent binding sites as described by the equation $R = R_{\max 1} \cdot C / (K_{d1} + C) + R_{\max 2} \cdot C / (K_{d2} + C)$).

- 10 The goodness-of-fits of these two models were analyzed visually by comparison with experimental data and statistically by an F test of the sums-of-squares. The simpler one-site model was chosen as the best fit, unless the two-site model fit significantly better ($p < 0.1$).

- 15 Association and disassociation analyses were performed using BIA evaluation 2.1 Software (Pharmacia). Association rate constants k_{on} were calculated in two ways, assuming both homogenous single-site interactions and parallel two-site interactions. For single-site interactions, k_{on} values were calculated according to the equation $R_t = R_{eq}(1 - \exp^{-k_s(t-t_0)})$, where R_t is a response at a given time, t ; R_{eq} is the steady-state response; t_0 is the time at the start of the injection; and $k_s = dR/dt = k_{on} \cdot C$, and where C is a concentration of analyte, calculated in terms of monomeric binding sites. For two-site interactions k_{on} values were calculated according to the equation $R_t = R_{eq1}(1 - \exp^{-k_{s1}(t-t_0)}) + R_{eq2}(1 - \exp^{-k_{s2}(t-t_0)})$. For each model, the values of k_{on} were determined from the calculated slope (to about 70% maximal association) of plots of k_s versus C .

- 25 Dissociation data were analyzed according to one site ($AB = A + B$) or two sites ($A_i B_j = A_i + B_j$) models, and rate constants (k_{off}) were calculated from best fit curves. The binding site model was used except when the residuals were greater than machine background (2-10 RU, according to machine), in which case the two-binding site model was employed. Half-times of receptor occupancy were calculated using the relationship
- 30 $t_{1/2} = 0.693 / k_{off}$.

Flow Cytometry:

Murine mAb L307.4 (anti-CD80) was purchased from Becton Dickinson (San Jose, California) and IT2.2 (anti-B7-0 [also known as CD86]), from Pharmingen (San Diego, California). For immunostaining, CD80-positive and/or CD86-positive CHO cells were removed from their culture vessels by incubation in phosphate-buffered saline (PBS) containing 10mM EDTA. CHO cells ($1-10 \times 10^5$) were first incubated with mAbs or immunoglobulin fusion proteins in DMEM containing 10% fetal bovine serum (FBS), then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-human immunoglobulin second step reagents (Tago, Burlingame, California). Cells were given a final wash and analyzed on a FACScan (Becton Dickinson).

SDS-PAGE and Size Exclusion Chromatography

SDS-PAGE was performed on Tris/glycine 4-20% acrylamide gels (Novex, San Diego, CA). Analytical gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning. CTLA4Ig (25 μ g) and L104EA29YIg (25 μ g) were analyzed by size exclusion chromatography using a TSK-GEL G300 SW_{XL} column (7.8 x 300mm, Tosohaas, Montgomeryville, PA) equilibrated in phosphate buffered saline containing 0.02% NaN₃ at a flow rate of 1.0 ml/min.

CTLA4X_{C120S} and L104EA29YX_{C120S}.

Single chain CTLA4X_{C120S} was prepared as previously described (Linsley et al., (1995) J. Biol. Chem., 270:15417-15424 (84)). Briefly, an oncostatin M CTLA4 (OMCTLA4) expression plasmid was used as a template, the forward primer,

25 GAGGTGATAAAGCTTCACCAATGGGTGTACTGCTCACACAG_A

was chosen to match sequences in the vector; and the reverse primer,

26 GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC_A

corresponded to the last seven amino acids (i.e. amino acids 118-124) in the extracellular domain of CTLA4, and contained a restriction enzyme site, and a stop codon (TGA).

30 The reverse primer specified a C120S (cysteine to serine at position 120) mutation. In particular, the nucleotide sequence GCA (nucleotides 34-36) of the reverse primer shown

above is replaced with one of the following nucleotide sequences: AGA, GGA, TGA, CGA, ACT, or GCT. As persons skilled in the art will understand, the nucleotide sequence GCA is a reversed complementary sequence of the codon TGC for cysteine. Similarly, the nucleotide sequences AGA, GGA, TGA, CGA, ACT, or GCT are the reversed complementary sequences of the codons for serine. Polymerase chain reaction products were digested with *HindIII/XbaI* and directionally subcloned into the expression vector π LN (Bristol-Myers Squibb Company, Princeton, NJ). L104EA29YX_{C120S} was prepared in an identical manner. Each construct was verified by DNA sequencing.

10 Identification and Biochemical Characterization of High Avidity Mutants

Twenty four amino acids were chosen for mutagenesis and the resulting ~2300 mutant proteins assayed for CD86Ig binding by surface plasmon resonance (SPR; as described, supra). The predominant effects of mutagenesis at each site are summarized in Table II. Random mutagenesis of some amino acids in the S25-R33 apparently did not alter ligand binding. Mutagenesis of E31 and R33 and residues M97-Y102 apparently resulted in reduced ligand binding. Mutagenesis of residues, S25, A29, and T30, K93, L96, Y103, L104, and G105, resulted in proteins with slow "on" and/or slow "off" rates. These results confirm previous findings that residues in the S25-R33 region, and residues in or near M97-Y102 influence ligand binding (Peach et al., (1994) J. Exp. Med., 180:2049-2058 (85)).

Mutagenesis of sites S25, T30, K93, L96, Y103, and G105 resulted in the identification of some mutant proteins that had slower "off" rates from CD86Ig. However, in these instances, the slow "off" rate was compromised by a slow "on" rate which resulted in mutant proteins with an overall avidity for CD86Ig that was apparently similar to that seen with wild type CTLA4Ig. In addition, mutagenesis of K93 resulted in significant aggregation which may have been responsible for the kinetic changes observed.

Random mutagenesis of L104 followed by COS cell transfection and screening by SPR of culture media samples over immobilized CD86Ig yielded six media samples containing mutant proteins with approximately 2-fold slower "off" rates than wild type

CTLA4Ig. When the corresponding cDNA of these mutants were sequenced, each was found to encode a leucine to glutamic acid mutation (L104E). Apparently, substitution of leucine 104 to aspartic acid (L104D) did not affect CD86Ig binding.

5 Mutagenesis was then repeated at each site listed in Table II, this time using L104E as the PCR template instead of wild type CTLA4Ig, as described above. SPR analysis, again using immobilized CD86Ig, identified six culture media samples from mutagenesis of alanine 29 with proteins having approximately 4-fold slower "off" rates than wild type CTLA4Ig. The two slowest were tyrosine substitutions (L104EA29Y), two were leucine
10 (L104EA29L), one was tryptophan (L104EA29W), and one was threonine (L104EA29T). Apparently, no slow "off" rate mutants were identified when alanine 29 was randomly mutated, alone, in wild type CTLA4Ig.

The relative molecular mass and state of aggregation of purified L104E and
15 L104EA29YIg was assessed by SDS-PAGE and size exclusion chromatography. L104EA29YIg (~1 µg; lane 3) and L104EIg (~1 µg; lane 2) apparently had the same electrophoretic mobility as CTLA4Ig (~1 µg; lane 1) under reducing (~50kDa; +βME; plus 2-mercaptoethanol) and non-reducing (~100kDa; -βME) conditions (FIG. 7A). Size exclusion chromatography demonstrated that L104EA29YIg (FIG. 7C) apparently had
20 the same mobility as dimeric CTLA4Ig (FIG. 7B). The major peaks represent protein dimer while the faster eluting minor peak in FIG. 7B represents higher molecular weight aggregates. Approximately 5.0% of CTLA4Ig was present as higher molecular weight aggregates but there was no evidence of aggregation of L104EA29YIg or L104EIg. Therefore, the stronger binding to CD86Ig seen with L104EIg and L104EA29YIg could
25 not be attributed to aggregation induced by mutagenesis.

Equilibrium and Kinetic Binding Analysis

Equilibrium and kinetic binding analysis was performed on protein A purified CTLA4Ig, L104EIg, and L104EA29YIg using surface plasmon resonance (SPR). The results are
30 shown in Table I. Observed equilibrium dissociation constants (K_d ; Table I) were calculated from binding curves generated over a range of concentrations (5.0-200 nM).

L104EA29YIg binds more strongly to CD86Ig than does L104EIg or CTLA4Ig. The lower K_d of L104EA29YIg (3.21 nM) than L104EIg (6.06 nM) or CTLA4Ig (13.9 nM) indicates higher binding avidity of L104EA29YIg to CD86Ig. The lower K_d of L104EA29YIg (3.66 nM) than L104EIg (4.47 nM) or CTLA4Ig (6.51 nM) indicates higher binding avidity of L104EA29YIg to CD80Ig.

Kinetic binding analysis revealed that the comparative "on" rates for CTLA4Ig, L104EIg, and L104EA29YIg binding to CD80 were similar, as were the "on" rates for CD86Ig (Table I). However, "off" rates for these molecules were not equivalent (Table I). Compared to CTLA4Ig, L104EA29YIg had approximately 2-fold slower "off" rate from CD80Ig, and approximately 4-fold slower "off" rate from CD86Ig. L104E had "off" rates intermediate between L104EA29YIg and CTLA4Ig. Since the introduction of these mutations did not significantly affect "on" rates, the increase in avidity for CD80Ig and CD86Ig observed with L104EA29YIg was likely primarily due to a decrease in "off" rates.

To determine whether the increase in avidity of L104EA29YIg for CD86Ig and CD80Ig was due to the mutations affecting the way each monomer associated as a dimer, or whether there were avidity enhancing structural changes introduced into each monomer, single chain constructs of CTLA4 and L104EA29Y extracellular domains were prepared following mutagenesis of cysteine 120 to serine as described *supra*, and by Linsley et al., (1995) *J. Biol. Chem.*, 270:15417-15424 (84). The purified proteins CTLA4X_{C120S} and L104EA29YX_{C120S} were shown to be monomeric by gel permeation chromatography (Linsley et al., (1995), *supra* (84)), before their ligand binding properties were analyzed by SPR. Results showed that binding affinity of both monomeric proteins for CD86Ig was approximately 35-80-fold less than that seen for their respective dimers (Table I). This supports previously published data establishing that dimerization of CTLA4 was required for high avidity ligand binding (Greene et al., (1996) *J. Biol. Chem.*, 271:26762-26771 (78)).

L104EA29YX_{C120S} bound with approximately 2-fold higher affinity than CTLA4X_{C120S} to both CD80Ig and CD86Ig. The increased affinity was due to approximately 3-fold slower rate of dissociation from both ligands. Therefore, stronger ligand binding by L104EA29Y was most likely due to avidity enhancing structural changes that had been introduced into each monomeric chain rather than alterations in which the molecule dimerized.

Location and Structural Analysis of Avidity Enhancing Mutations

10 The solution structure of the extracellular IgV-like domain of CTLA4 has recently been determined by NMR spectroscopy (Metzler et al., (1997) *Nature Struct. Biol.*, 4:527-531 (86)). This allowed accurate location of leucine 104 and alanine 29 in the three dimensional fold (FIG. 8A-B). Leucine 104 is situated near the highly conserved MYPPPY amino acid sequence. Alanine 29 is situated near the C-terminal end of the
15 S25-R33 region, which is spatially adjacent to the MYPPPY region. While there is significant interaction between residues at the base of these two regions, there is apparently no direct interaction between L104 and A29 although they both comprise part of a contiguous hydrophobic core in the protein. The structural consequences of the two avidity enhancing mutants were assessed by modeling. The A29Y mutation can be easily
20 accommodated in the cleft between the S25-R33 region and the MYPPPY region, and may serve to stabilize the conformation of the MYPPPY region. In wild type CTLA4, L104 forms extensive hydrophobic interactions with L96 and V94 near the MYPPPY region. It is highly unlikely that the glutamic acid mutation adopts a conformation similar to that of L104 for two reasons. First, there is insufficient space to accommodate
25 the longer glutamic acid side chain in the structure without significant perturbation to the S25-R33 region. Second, the energetic costs of burying the negative charge of the glutamic acid side chain in the hydrophobic region would be large. Instead, modeling studies predict that the glutamic acid side chain flips out on to the surface where its charge can be stabilized by solvation. Such a conformational change can easily be
30 accommodated by G105, with minimal distortion to other residues in the regions.

Binding of High Avidity Mutants to CHO Cells Expressing CD80 or CD86

FACS analysis (Fig. 9) of CTLA4Ig and mutant molecules binding to stably transfected CD80+ and CD86+CHO cells was performed as described herein. CD80-positive and CD86-positive CHO cells were incubated with increasing concentrations of CTLA4Ig, L104EA29YIg, or L104EIg, and then washed. Bound immunoglobulin fusion protein was detected using fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin.

As shown in Figure 9, CD80-positive or CD86-positive CHO cells (1.5×10^5) were incubated with the indicated concentrations of CTLA4Ig (closed squares), L104EA29YIg (circles), or L104EIg (triangles) for 2 hr. at 23°C, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin antibody. Binding on a total of 5,000 viable cells was analyzed (single determination) on a FACScan, and mean fluorescence intensity (MFI) was determined from data histograms using PC-LYSYS. Data were corrected for background fluorescence measured on cells incubated with second step reagent only (MFI = 7). Control L6 mAb (80 µg/ml) gave MFI < 30. These results are representative of four independent experiments.

Binding of L104EA29YIg, L104EIg, and CTLA4Ig to human CD80-transfected CHO cells is approximately equivalent (FIG. 9A). L104EA29YIg and L104EIg bind more strongly to CHO cells stably transfected with human CD86 than does CTLA4Ig (FIG. 9B).

Functional Assays:

Human CD4-positive T cells were isolated by immunomagnetic negative selection (Linsley et al., (1992) *J. Exp. Med.* 176:1595-1604 (83)). Isolated CD4-positive T cells were stimulated with phorbol myristate acetate (PMA) plus CD80-positive or CD86-positive CHO cells in the presence of titrating concentrations of inhibitor. CD4-positive T cells ($8-10 \times 10^4$ /well) were cultured in the presence of 1 nM PMA with or without

irradiated CHO cell stimulators. Proliferative responses were measured by the addition of 1 μ Ci/well of [3H]thymidine during the final 7 hours of a 72 hour culture. Inhibition of PMA plus CD80-positive CHO, or CD86-positive CHO, stimulated T cells by L104EA29YIg and CTLA4Ig was performed. The results are shown in FIG. 10.

5 L104EA29YIg inhibits proliferation of CD80-positive PMA treated CHO cells more than CTLA4Ig (FIG. 10A). L104EA29YIg is also more effective than CTLA4Ig at inhibiting proliferation of CD86-positive PMA treated CHO cells (FIG. 10B). Therefore, L104EA29YIg is a more potent inhibitor of both CD80- and CD86-mediated costimulation of T cells.

10

Figure 11 shows inhibition by L104EA29YIg and CTLA4Ig of allostimulated human T cells prepared above, and further allostimulated with a human B lymphoblastoid cell line (LCL) called PM that expressed CD80 and CD86 (T cells at 3.0×10^4 /well and PM at 8.0×10^3 /well). Primary allostimulation occurred for 6 days, then the cells were pulsed with 3 H-thymidine for 7 hours, before incorporation of radiolabel was determined.

15

Secondary allostimulation was performed as follows. Seven day primary allostimulated T cells were harvested over lymphocyte separation medium (LSM) (ICN, Aurora, OH) and rested for 24 hours. T cells were then restimulated (secondary), in the presence of titrating amounts of CTLA4Ig or L104EA29YIg, by adding PM in the same ratio as above. Stimulation occurred for 3 days, then the cells were pulsed with radiolabel and harvested as above. The effect of L104EA29YIg on primary allostimulated T cells is shown in FIG. 11A. The effect of L104EA29YIg on secondary allostimulated T cells is shown in FIG. 11B. L104EA29YIg inhibits both primary and secondary T cell proliferative responses better than CTLA4Ig.

20

25

To measure cytokine production (Figure 12), duplicate secondary allostimulation plates were set up. After 3 days, culture media was assayed using ELISA kits (Biosource, Camarillo, CA) using conditions recommended by the manufacturer. L104EA29YIg was found to be more potent than CTLA4Ig at blocking T cell IL-2, IL-4, and γ -IFN cytokine production following a secondary allogeneic stimulus (FIGS. 12A-C).

30

The effects of L104EA29YIg and CTLA4Ig on monkey mixed lymphocyte response (MLR) are shown in Figure 13. Peripheral blood mononuclear cells (PBMC'S; 3.5×10^4 cells/well from each monkey) from 2 monkeys were purified over lymphocyte separation medium (LSM) and mixed with $2 \mu\text{g/ml}$ phytohemagglutinin (PHA). The cells were stimulated 3 days then pulsed with radiolabel 16 hours before harvesting. L104EA29YIg inhibited monkey T cell proliferation better than CTLA4Ig.

Table I:

Equilibrium and apparent kinetic constants are given in the following table (values are means \pm standard deviation from three different experiments):

Immobilized Protein	Analyte	$k_{on} (x 10^5) M^{-1} S^{-1}$	$k_{off} (x 10^{-3}) S^{-1}$	K_d nM
CD80Ig	CTLA4Ig	3.44 ± 0.29	2.21 ± 0.18	6.51 ± 1.08
CD80Ig	L104EIg	3.02 ± 0.05	1.35 ± 0.08	4.47 ± 0.36
CD80Ig	L104EA29YIg	2.96 ± 0.20	1.08 ± 0.05	3.66 ± 0.41
CD80Ig	CTLA4X _{C120S}	12.0 ± 1.0	230 ± 10	195 ± 25
CD80Ig	L104EA29YX _{C120S}	8.3 ± 0.26	71 ± 5	85.0 ± 2.5
CD86Ig	CTLA4Ig	5.95 ± 0.57	8.16 ± 0.52	13.9 ± 2.27
CD86Ig	L104EIg	7.03 ± 0.22	4.26 ± 0.11	6.06 ± 0.05
CD86Ig	L104EA29YIg	6.42 ± 0.40	2.06 ± 0.03	3.21 ± 0.23
CD86Ig	CTLA4X _{C120S}	16.5 ± 0.5	840 ± 55	511 ± 17
CD86Ig	L104EA29YX _{C120S}	11.4 ± 1.6	300 ± 10	267 ± 29

Table II

The effect on CD86Ig binding by mutagenesis of CTLA4Ig at the sites listed was determined by SPR, described supra. The predominant effect is indicated with a "+" sign.

Mutagenesis Site	Effects of Mutagenesis				
	No Effect	Apparent	Slow "on" rate/ "off rate	Reduced binding	ligand
S25			+		
P26	+				
G27	+				
K28	+				
A29			+		
T30			+		
E31				+	
R33				+	
K93			+		
L96			+		
M97				+	
Y98				+	
P99				+	
P100				+	
P101				+	
Y102				+	
Y103			+		
L104			+		
G105			+		
I106	+				
G107	+				
Q111	+				
Y113	+				
I115	+				

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